REFERENCES

Alvarez, C.E. (2008). BMC Evol. Biol. *8*, 222.

Belgareh-Touze, N., Leon, S., Erpapazoglou, Z., Stawiecka-Mirota, M., Urban-Grimal, D., and Haguenauer-Tsapis, R. (2008). Biochem. Soc. Trans. *36*, 791–796.

DeWire, S.M., Ahn, S., Lefkowitz, R.J., and Shenoy, S.K. (2007). Annu. Rev. Physiol. *69*, 483–510.

Lin, C.H., MacGurn, J.A., Chu, T., Stefan, C.J., and Emr, S.D. (2008). Cell, this issue.

Mukhopadhyay, D., and Riezman, H. (2007). Science *315*, 201–205.

Nikko, E., Sullivan, J.A., and Pelham, H.R. (2008). EMBO Rep. Published online October 24, 2008. 10.1038/embor.2008.199.

Peng, J., Schwartz, D., Elias, J.E., Thoreen, C.C., Cheng, D., Marsischky, G., Roelofs, J., Finley, D., and Gygi, S.P. (2003). Nat. Biotechnol. *21*, 921–926.

Shenoy, S.K., Xiao, K., Venkataramanan, V., Snyder, P.M., Freedman, N.J., and Weissman, A.M. (2008). J. Biol. Chem. *283*, 22166–22176.

Wiesner, S., Ogunjimi, A.A., Wang, H.R., Rotin, D., Sicheri, F., Wrana, J.L., and Forman-Kay, J.D. (2007). Cell *130*, 651–662.

Pol V Transcribes to Silence

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DOI 10.1016/j.cell.2008.10.027

In most cases, the functions of long noncoding RNAs remain uncertain. Working in the model plant *Arabidopsis*, Wierzbicki et al. (2008) provide evidence that transcription of intergenic noncoding regions by RNA polymerase V promotes heterochromatin formation and silencing of nearby genes.

A group of unusual RNA polymerase subunits has intrigued plant biologists ever since their discovery during the initial analysis of the *Arabidopsis* genome. In addition to DNA-dependent RNA polymerases I, II, and III, flowering plants have two extra RNA polymerases termed Pol IV and Pol V. Genetic experiments have implicated Pol IV and Pol V in chromatin-based gene silencing mediated by small RNAs (Pikaard et al., 2008), and current models suggest that the two polymerase complexes act at different steps of the silencing pathway: Pol IV produces and amplifies the small RNA trigger, whereas Pol V functions downstream to facilitate de novo DNA methylation at the site targeted by the small RNA. Although Pol IV is involved in the production of 24 nucleotide (nt) "heterochromatic" small-interfering RNAs (siRNAs) (Mosher et al., 2008), the function of Pol V in gene silencing is unclear. For instance, it is unknown whether Pol V transcribes extensively or simply opens chromatin at the siRNA-targeted site to expose DNA to cytosine methyltransferases (Kanno et al., 2005).

A study published in this issue of *Cell* (Wierzbicki et al., 2008) provides the first evidence of transcripts generated by Pol V and documents their role in chromatin-based gene silencing. The newly discovered Pol V transcripts originate from intergenic noncoding regions and facilitate siRNA-directed epigenetic modifications that impede transcription of overlapping and neighboring transposons and genes by Pol II and Pol III. The findings nicely illustrate how plants have diversified their transcriptional machinery to include specialized RNA polymerases that participate in the formation of repressed chromatin for gene silencing.

Prior work in fission yeast has shown that RNAi-mediated formation of heterochromatin depends, paradoxically, on Pol II transcription of the target sequence. The Pol II-generated noncoding RNAs have a dual function in heterochromatin assembly, serving both as precursors for siRNAs and as scaffolds that interact with siRNAs to recruit chromatin-modifying factors. A JmjC domain-containing protein, Epe1, enables Pol II transcription of heterochromatic repeats by counteracting repressed chromatin (Zofall and Grewal, 2006). Given the precedent for Pol II-generated noncoding RNAs in siRNA-mediated heterochromatin formation in fission yeast, Wierzbicki and coworkers sought to determine whether Pol IV and/or Pol V transcribe noncoding RNAs in plants.

The authors first suspected Pol V transcription from their inspection of the heterochromatic knob on *Arabidopsis* chromosome 4. Although this region is hypermethylated and encodes numerous siRNAs, certain areas are devoid of detectable RNA transcripts on DNA tiling arrays. Working on the hypothesis that low abundance transcripts might provide precursors for siRNAs, they used RT-PCR to look for intergenic noncoding RNAs that disappear in plants lacking Pol IV or Pol V. Of 14 regions examined, 6 gave rise to RNAs that were lost only in plants deficient in Pol V. Four intergenic noncoding transcripts were derived from transposon and repeat-rich regions, whereas two originated from gene-rich portions of the genome, indicating that Pol V transcribes in both

heterochromatic and euchromatic environments. The Pol V-dependent transcripts are at least 200 nt in size, initiate from multiple sites, have triphosphates or 7-methylguanosine caps at their 5′ ends, and lack poly A tails.

The largest subunits of Pol IV and Pol V are nuclear RNA polymerase D (NRPD1) and nuclear RNA polymerase E (NRPE1), respectively; NRPD1 and NRPE1 evolved successively from the largest subunit of Pol II (RPB1) (Luo and Hall, 2007). Wierzbicki et al. obtained evidence that Pol V directly synthesizes intergenic noncoding transcripts by using an *nrpe1* mutant of *Arabidopsis* with an amino acid substitution in the active site. Both NRPD1 and NRPE1 have an invariant DFDGD motif at the active site (metal A site) that coordinates a magnesium ion that is required for nucleoside polymerization. The importance of the metal A site was assessed by analyzing *nrpe1* mutants transformed with a wild-type *NRPE1* transgene or an *NRPE1* transgene in which the invariant aspartates were changed to alanine. Only the wild-type *NRPE1* transgene restored the transcription of the intergenic noncoding regions by Pol V in an *nrpe1* mutant. Chromatin immunoprecipitation experiments using FLAG-tagged versions of NRPE1 and NRPB2 (the second largest subunit of Pol II) showed that NRPE1 physically associates with loci that encode intergenic noncoding transcripts and with the transcripts themselves.

Additional experiments demonstrated that Pol V transcription is required to silence Pol II and Pol III transcription of overlapping and adjacent sequences. Derepression of Pol II transcription in regions flanking a retrotransposon solo long-terminal repeat (LTR) occurred only in the absence of Pol V transcription of solo LTR sequences in the *nrpe1* mutant. Similarly, Pol III-generated transcripts of the SINE element *AtSN1* were detectable only if Pol V transcription was abolished in the *nrpe1* mutant (Figure 1). Pol V transcription is needed to induce heterochromatic marks at affected loci, including DNA and histone methylation. Locus-specific changes in repressive modifications were observed, which may reflect differences in sequence composition and location in euchromatin or heterochromatin.

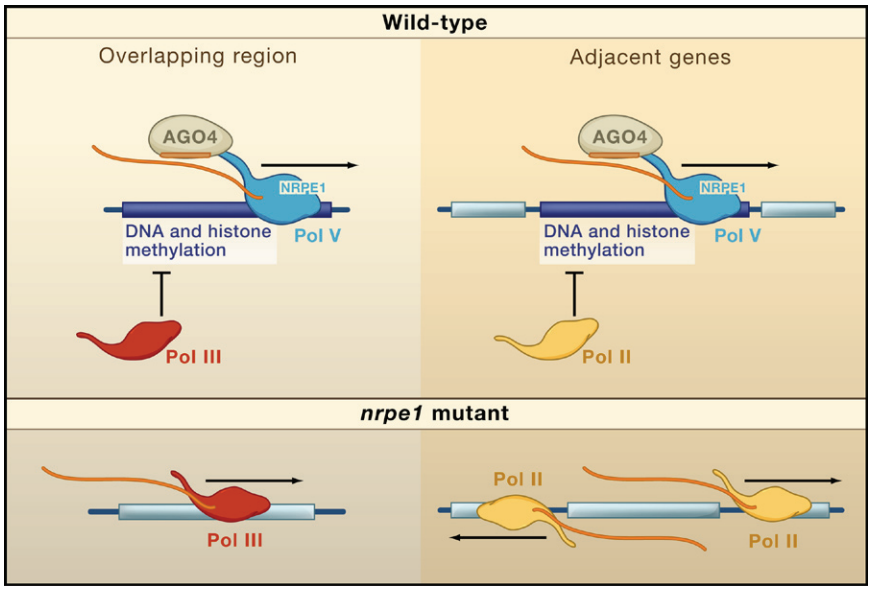

Figure 1. Transcription by *Arabidopsis* Pol V Silences Overlapping and Adjacent Sequences
In wild-type plants, Pol V transcription of intergenic regions triggers DNA and histone methylation, which blocks transcription of overlapping or adjacent genes and transposons (blue rectangles) by Pol II and Pol III. Interactions between the Pol V nascent RNA (orange line) and siRNAs (brown bar) bound to AGO4, which interacts with the C-terminal domain of NRPE1 (the largest subunit of Pol V), provide sequence specificity and may serve as a scaffold for attracting DNA methyltransferases and histone methyltransferases (not shown). In an *nrpe1* mutant, Pol V-generated transcripts disappear and methylation is lost, allowing uni- and/or bidirectional transcription by Pol II and Pol III.

Wierzbicki and coworkers have provided convincing evidence for Pol V transcription of intergenic noncoding RNAs that elicit repressive epigenetic modifications and induce silencing of overlapping and neighboring sequences. The results help to resolve the paradoxical requirement for transcription to establish and maintain RNA-mediated chromatin-based silencing. However, Pol V transcription alone is not sufficient for silencing, which also depends on Pol IV-generated siRNAs. Wierzbicki and coworkers suggest three models for how Pol V transcripts and siRNAs act in this pathway. The favored one posits interactions between the Pol V-generated nascent RNA and ARGONAUTE4 (AGO4)-bound siRNAs. This model resembles the mechanism proposed for RNAi-mediated heterochromatin assembly in fission yeast. However, it may be premature to limit Pol V action to a single mechanism. Kanno et al. (2005) isolated a plant bearing a mutation in the active site of NRPE1 in a forward genetics screen for mutants defective in siRNA-directed DNA methylation of a transgene promoter. However, transcripts associated with the transgene promoter have not been detected in this mutant (T. Kanno and M.M., unpublished data). Thus, Pol V transcripts may not always play a direct role in triggering epigenetic modifications. An alternative hypothesis, also suggested by Wierzbicki and coworkers, is that Pol V initiation, but not elongation, opens chromatin to allow siRNA-DNA base pairing at the target site.

The new results suggest a function for the pervasive intergenic transcription detected in eukaryotic genomes (Kapronov et al., 2007), and they open the door to an extended genome-wide analysis of Pol V transcripts in *Arabidopsis*. Pol IV and Pol V substantially enhance the ability of plants to modulate the epigenetic state of their genomes during transcription. Similar to the C-terminal domain of the Pol II subunit RPB1, which recruits enzymes catalyzing histone modifications associated with genes actively transcribed by Pol II (Egloff and Murphy, 2008), the unique C-terminal domains of NRPE1 and NRPD1 are likely to attract factors

that perform roles specific to silencing. AGO4, for example, interacts with WG/GW repeats in the C-terminal domain of NRPE1 (El-Shami et al., 2007). In addition to establishing and maintaining repressive modifications, siRNAs may also guide active demethylation of DNA to reverse silencing (Zheng et al., 2008). Plants have thus elaborated nuclear pathways of siRNA-mediated silencing to promote epigenetic flexibility that may be important for stress adaptation and developmental plasticity.

REFERENCES

Egloff, S., and Murphy, S. (2008). Trends Genet. *24*, 280–288.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.A., Jacobsen, S.E., Cooke, R., and Lagrange, T. (2007). Genes Dev. *21*, 2539–2544.

Kanno, T., Huettel, B., Mette, M.F., Aufsatz, W., Jaligot, E., Daxinger, L., Kreil, D.P., Matzke, M., and Matzke, A.J.M. (2005). Nat. Genet. *37*, 761–765.

Kapronov, P., Willingham, A.T., and Gingeras, T.R. (2007). Nat. Rev. Genet. *8*, 413–423.

Luo, J., and Hall, B.D. (2007). J. Mol. Evol. *64*, 101–112.

Mosher, R.A., Schwach, F., Studholme, D., and Baulcombe, D.C. (2008). Proc. Natl. Acad. Sci. USA *105*, 3145–3150.

Pikaard, C.S., Haag, J.R., Ream, T., and Wierzbicki, A.T. (2008). Trends Plant Sci. *13*, 390–397.

Wierzbicki, A.T., Haag, J.R., and Pikaard, C.S. (2008). Cell, this issue.

Zheng, X., Pontes, O., Zhu, J., Miki, D., Zhang, F., Li, W.X., Iida, K., Kapoor, A., Pikaard, C.S., and Zhu, J.K. (2008). Nature. Published online September 24, 2008. 10.1038/nature07305.

Zofall, M., and Grewal, S.I.S. (2006). Mol. Cell *22*, 681–692.

The Safety on the TCR Trigger

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DOI 10.1016/j.cell.2008.10.033

In this issue, Xu et al. (2008) provide evidence for a new mechanism of T cell receptor regulation. Prior to activation, basic residues in the cytoplasmic domain of the signaling subunits of the T cell receptor associate with the plasma membrane such that the key signaling tyrosines are sequestered in the bilayer.

In order to sense the presence of foreign invaders, many cells of the innate and adaptive immune systems employ activating immune receptors that are comprised of a ligand-binding subunit with no signaling capacity and subunits that couple the receptor to the intracellular signaling machinery (Call and Wucherpfennig, 2007). A key part of the signaling capacity of these receptors resides in immunoreceptor tyrosine-based activation motifs (ITAMs) (Reth, 1989). Of the many receptors that contain ITAMs, the one used by αβT cells to recognize antigen is the most complicated. The α and β chains of the heterodimeric T cell receptor (TCR) typically bind the composite surface of a peptide embedded within a major histocompatibility complex molecule (pMHC). Information about the potency of the pMHC is then transferred to the ITAMs of the associated CD3γε, CD3δε, and CD3ζζ signaling dimers, which are subsequently phosphorylated by the Src kinases, Lck and Fyn. This process is commonly referred to as TCR triggering; it is a complicated process that remains poorly understood despite the efforts of many investigators over more than 20 years. The results of a collaborative effort from the Chou and Wucherpfennig labs, presented in this issue (Xu et al., 2008), represent an important breakthrough in defining a mechanism by which interactions of ITAMs with the plasma membrane act as the "safety" on the TCR trigger.

A fully assembled TCR-CD3 complex contains a total of ten ITAMs: one each for CD3γ, δ, and ε and three in CD3ζ. Experiments in which the ITAMs have been selectively inactivated indicate that the number of ITAMs that are phosphorylated upon TCR engagement determines how thymocytes or mature T cells respond (Holst et al., 2008). Thus, having ten ITAMs is thought to allow for "scalable signaling" to elicit the appropriate thymocyte or T cell response from a range of potential responses that can result from TCR engagement.

This suggests that tight control over ITAM phosphorylation must exist to prevent inappropriate signaling. Aivazian and Stern (2000) showed that a recombinant cytoplasmic domain of CD3ζ, which has a net positive charge due to clusters of basic amino acids, can bind to acidic phospholipid vesicles and that this interaction prevents phosphorylation of the CD3ζ ITAMs by Lck. They proposed a model in which the binding of the CD3ζ cytoplasmic domain to the plasma membrane in some way prevents the spontaneous phosphorylation of the ITAMs, whereas receptor multimerization, upon TCR engagement, results in a high local concentration of the intracellular domains.